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Aspirin Exposure Reveals Novel Genes Associated With Platelet Function and Cardiovascular Events

Deepak Voora, MD,*† Derek Cyr, PHD,* Joseph Lucas, PHD,* Jen-Tsan Chi, MD, PHD,* Jennifer Dungan, PHD,† Timothy A. McCaffrey, PHD,‡ Richard Katz, MD,§ L. Kristin Newby, MD, MHS,† William E. Kraus, MD,† Richard C. Becker, MD,† Thomas L. Ortel, MD, PHD,† Geoffrey S. Ginsburg, MD, PHD*†

Durham, North Carolina; and Washington, DC

Objectives The aim of this study was to develop ribonucleic acid (RNA) profiles that could serve as novel biomarkers for the response to aspirin.

Background Aspirin reduces death and myocardial infarction (MI), suggesting that aspirin interacts with biological pathways that may underlie these events.

Methods Aspirin was administered, followed by whole-blood RNA microarray profiling, in a discovery cohort of healthy volunteers (HV1) (n = 50) and 2 validation cohorts of healthy volunteers (HV2) (n = 53) and outpatient cardiology patients (OPC) (n = 25). Platelet function was assessed using the platelet function score (PFS) in HV1 and HV2 and the VerifyNow Aspirin Test (Accumetrics, Inc., San Diego, California) in OPC. Bayesian sparse factor analysis identified sets of coexpressed transcripts, which were examined for associations with PFS in HV1 and validated in HV2 and OPC. Proteomic analysis confirmed the association of validated transcripts in platelet proteins. Validated gene sets were tested for association with death or MI in 2 patient cohorts (n = 587 total) from RNA samples collected at cardiac catheterization.

Results A set of 60 coexpressed genes named the "aspirin response signature" (ARS) was associated with PFS in HV1 ($r = -0.31$, $p = 0.03$), HV2 ($r = -0.34$, Bonferroni $p = 0.03$), and OPC ($p = 0.046$). Corresponding proteins for the 17 ARS genes were identified in the platelet proteome, of which 6 were associated with PFS. The ARS was associated with death or MI in both patient cohorts (odds ratio: 1.2 [$p = 0.01$]; hazard ratio: 1.5 [$p = 0.001$]), independent of cardiovascular risk factors. Compared with traditional risk factors, reclassification (net reclassification index = 31% to 37%, $p \leq 0.0002$) was improved by including the ARS or 1 of its genes, *ITGA2B*.

Conclusions RNA profiles of platelet-specific genes are novel biomarkers for identifying patients who do not respond adequately to aspirin and who are at risk for death or MI. (J Am Coll Cardiol 2013;62:1267–76) © 2013 by the American College of Cardiology Foundation

The identification of novel biomarkers for patients at risk for coronary artery disease (CAD) mortality, primarily because of platelet-mediated cardiovascular events such as

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From the *Institute for Genome Sciences & Policy, Duke University, Durham, North Carolina; †Department of Medicine, Duke University, Durham, North Carolina; and the ‡Division of Genomic Medicine, The George Washington University/Medical Faculty Associates, Washington, DC; and the §Division of Cardiology, Department of Medicine, The George Washington University/Medical Faculty Associates, Washington, DC. This study was funded by institutional funds provided by the Duke Institute for Genome Sciences & Policy, National Institutes of Health (NIH) T32 training grant 5T32HL007101 to Dr. Voora, grant 5UL1RR024128 from the National Center for Research Resources, a component of the NIH, and the NIH Roadmap for Medical Research, grant 5RC1GM091083 to Dr. Ginsburg from the National Institutes of General Medical Sciences, grant 5U01DD000014 to Dr. Ortel from the Centers for Disease Control and Prevention, and the David H. Murdock Research Institute. Dr. Ginsburg is a consultant to United States Diagnostic Standards; is a scientific advisor to CardioDx, Pappas Ventures, and Universal Medicine; and holds equity in CardioDx. Dr. McCaffrey holds equity in Cellgenex. Dr. Newby has received research grants or contracts from Amylin, Inc., Bristol-Myers Squibb, Glaxo-SmithKline, Merck & Company, the MURDOCK Study, and the National Heart, Lung, and Blood Institute and provides consulting or other services to Daiichi-Sankyo, Genentech, Novartis, Roche Diagnostics, Jansen Pharmaceuticals, Inc., Navigant, and DSI-Lilly. Drs. Voora, Lucas, Chi, Becker, Ortel, and Ginsburg have filed a provisional patent application regarding the aspirin response signature. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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Abbreviations and Acronyms

ARS = aspirin response signature
CAD = coronary artery disease
CATHGEN = Catheterization Genetics
CI = confidence interval
DUMC = Duke University Medical Center
HV1 = healthy volunteer discovery cohort
HV2 = healthy volunteer validation cohort
MI = myocardial infarction
MPV = mean platelet volume
OPC = outpatient cardiology cohort
OR = odds ratio
PCR = polymerase chain reaction
PFS = platelet function score
RNA = ribonucleic acid
RT-PCR = real-time polymerase chain reaction

myocardial infarction (MI), is a priority for reducing the burden of cardiovascular disease. Although genomewide surveys of genomic variation and gene expression can identify loci associated with CAD (1–3), few can serve as biomarkers for cardiovascular events (4).

Aspirin is prescribed for the prevention of cardiovascular events, suggesting that aspirin interacts with biological pathways that may underlie these events. Platelet function assays are a surrogate biomarker for the effects of aspirin and are associated with cardiovascular events (5). However, platelet function testing is not widely available, primarily because of technical complexity. By contrast, whole-blood ribonucleic acid (RNA) profiling using polymerase chain reaction (PCR)–based assays is currently a widely available diagnostic testing platform (6,7). Therefore, we hypothesized that aspirin could be used as a probe in conjunction with whole-blood RNA profiling to elucidate novel biomarkers for platelet function in response to aspirin and for cardiovascular outcomes.

Methods

Platelet function outcomes in healthy volunteer cohorts at Duke University Medical Center. We previously described (8) the healthy volunteer discovery cohort (HV1) and the healthy volunteer validation cohort (HV2) (Online Fig. 1) and the platelet function score (PFS), a composite metric of the following platelet function assays: PFA-100 (collagen/epinephrine; Siemens Healthcare, Erlangen, Germany) closure time and the areas under the optical aggregometry curve induced by adenosine diphosphate (10, 5, and 1 μmol/l), epinephrine (10, 1, and 0.5 μmol/l), and collagen (5 and 2 mg/ml). We measured the PFS and mean platelet volume (MPV) in HV1 (n = 50) after 2 weeks of dosing with 325 mg/day non–enteric-coated, immediate-release aspirin and HV2 (n = 53) after 4 weeks of dosing with 325 mg/day aspirin. In both cohorts, whole-blood RNA was collected into PAXgene Blood RNA tubes (Becton Dickinson and Company, Franklin Lakes, New Jersey) before and after aspirin exposure and stored at −80°C until microarray profiling. Platelet count was measured in platelet-rich plasma in HV1.

Because 3 subjects in HV2 had participated in HV1, they were dropped from HV2, leaving 50 unique HV2 subjects. The Duke University Medical Center (DUMC) institutional review board approved the study protocols.

Platelet function outcomes in patients at risk for cardiovascular events at George Washington University. We previously described (9) the outpatient cardiology cohort (OPC) (Online Fig. 1), treated with 81 mg/day aspirin and assessed using the VerifyNow Aspirin Test (Accumetrics, Inc., San Diego, California) and whole-blood RNA microarray analysis.

Clinical outcomes in DUMC patients. CATHETERIZATION GENETICS BIOREPOSITORY. The Catheterization Genetics (CATHGEN) biorepository has banked, whole-blood RNA in PAXgene tubes from DUMC patients from the time of cardiac catheterization, baseline medical history, and follow-up for all-cause death and MI (10,11). Two cohorts had available microarray data (Online Fig. 2): in the observational cohort, 224 sequential samples were selected for RNA analysis, of which 191 had sufficient RNA for microarray analysis, and the case-control cohort consisted of participants who had experienced death or MI (n = 250) after their index catheterization and age-matched, sex-matched, and race-matched controls (n = 250) who were free of death or MI for >2 years after cardiac catheterization (12). Four hundred forty-seven had sufficient RNA for microarray analysis; 44 overlapped with the observational cohort and were dropped, leaving 403 subjects for analysis.

Follow-up for death and MI was ascertained in both cohorts in October 2011; the median follow-up duration was 3.8 years. Patients with incomplete follow-up were censored at the time of last contact. Patients who had histories of cardiac transplantation at the time of catheterization (n = 5), died within 7 days (n = 1), or failed quality control (n = 1) were excluded. The remaining datasets left 190 samples in the observational cohort (48 death or MI events) and 397 (202 death or MI events) in the case-control cohort.

RNA extraction, labeling, microarray hybridization, quality control, and normalization. See the Online Appendix for full details. Two microarray platforms were used: the Affymetrix U133A2 array (HV1, before aspirin; Affymetrix, Santa Clara, California) and the U133 Plus 2.0 array (all others). The robust multichip average method was used for normalization.

Real-time PCR. See the Online Appendix for full details. Forty-five transcripts were selected for verification in the original RNA samples on the basis of 2 criteria: 1) the strength of correlation of the probe set with PFS; and 2) the strength of membership between the probe set and the set of coexpressed genes of interest.

Platelet purification, protein sample preparation, and proteomics analysis by liquid chromatography–mass spectrometry/mass spectrometry. See the Online Appendix for full details.

Statistical analysis. The raw and normalized microarray data are available in the Gene Expression Omnibus for the OPC cohort (GSE38511). The data for the HV1, HV2, and CATHGEN cohorts are available through the Database of Genotypes and Phenotypes (phs000548.v1.p1 and phs000551.v1.p1). Unless stated otherwise, all tests were 2

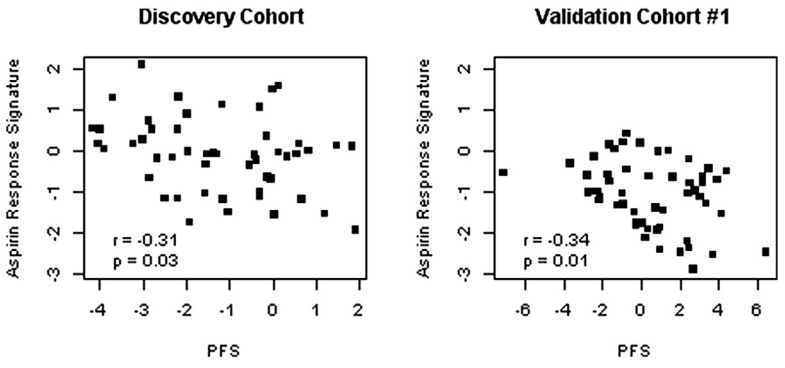

Figure 1 **The Aggregate Expression of a Set of Coexpressed Whole-Blood Genes Correlates With Platelet Function in Response to Aspirin**

Two independent cohorts of healthy volunteers were exposed to 325 mg/day aspirin, followed by whole-blood microarray profiling. Platelet function was assessed by the platelet function score (PFS) (8). The aggregate expression of a set of coexpressed genes (aspirin response signature [ARS], x-axis) is plotted against the PFS (y-axis) after aspirin exposure. Pearson's correlation coefficients and p values are reported.

sided and were performed using R version 2.10.0 (R Foundation for Statistical Computing, Vienna, Austria) or MATLAB version R2010b (The MathWorks, Natick, Massachusetts); p values <0.05 were considered significant.

DISCOVERY OF COEXPRESSED GENE SETS ASSOCIATED WITH PFS: FACTOR MODELING. The HV1, post-aspirin robust multichip average normalized data were nonspecifically filtered (i.e., without regard to PFS) to remove probes with mean expression <2.0 (i.e., the gene was not expressed in whole blood) or with variance <0.25 (i.e., the gene was homogenously expressed), resulting in 2,929 probe sets for subsequent analysis. To discover "factors," or sets of coexpressed genes representative of biological pathways, we used Bayesian factor regression modeling (13,14) in an unsupervised fashion (i.e., without regard to PFS). Each of the probe sets used to estimate a particular factor can be interpreted as a measurement of the activity of some (potentially unknown) biological pathway. Each sample can then be assigned a "factor score," which represents the aggregate expression of the transcripts within a factor. The factor scores can then be used for association with the phenotype of interest in subsequent analyses.

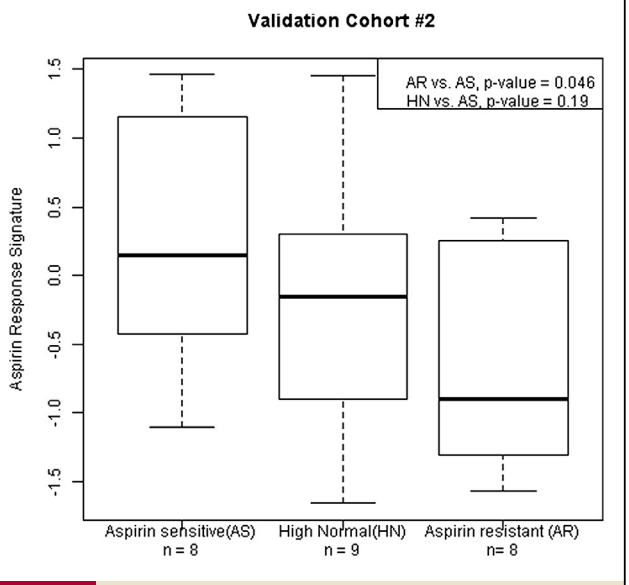

Figure 2 **The ARS Is Associated With Platelet Function in Patients at Risk for Cardiovascular Disease**

Patients treated with 81 mg/day aspirin were assessed using the VerifyNow Aspirin Test (9). Three categories of patients were profiled by microarray on the basis of their aspirin response units (ARU): aspirin resistant (AR; ARU >550), high normal (HN; 500 < ARU <550), and aspirin sensitive (AS; ARU <550). Aspirin response signature (ARS) values for each group are plotted and compared using 2-sample Student *t* tests. p values are 1-sided.

FACTOR PROJECTION, GENE MEMBERSHIP WITHIN A FACTOR, COMPARISON OF FACTOR GENE LISTS WITH SELECTED GENE SETS, AND COEXPRESSION OF TRANSCRIPTS REPRESENTED BY A FACTOR BEFORE AND AFTER ASPIRIN EXPOSURE. See the Online Appendix for full details.

CORRELATIONS BETWEEN FACTOR SCORES AND PLATELET FUNCTION. Pearson's correlation analysis was used to test for association between a factor and PFS in HV1 and HV2. In the second validation cohort, OPC, we chose a 1-sided Student *t* test because we hypothesized a lower factor score in the aspirin-resistant versus aspirin-sensitive groups.

Linear regression was used to assess the independent association of factor scores and PFS after accounting for log-transformed MPV and/or platelet count.

CORRECTION FOR MULTIPLE HYPOTHESIS TESTING. Because HV1 was a hypothesis-generating pilot study, we did not adjust p values. In the first validation cohort, HV2, we adjusted p values using Bonferroni correction. In the second validation cohort, we performed only 1 hypothesis test.

ANALYSES OF REAL-TIME PCR DATA. The expression of each selected transcript relative to the 3 reference genes was

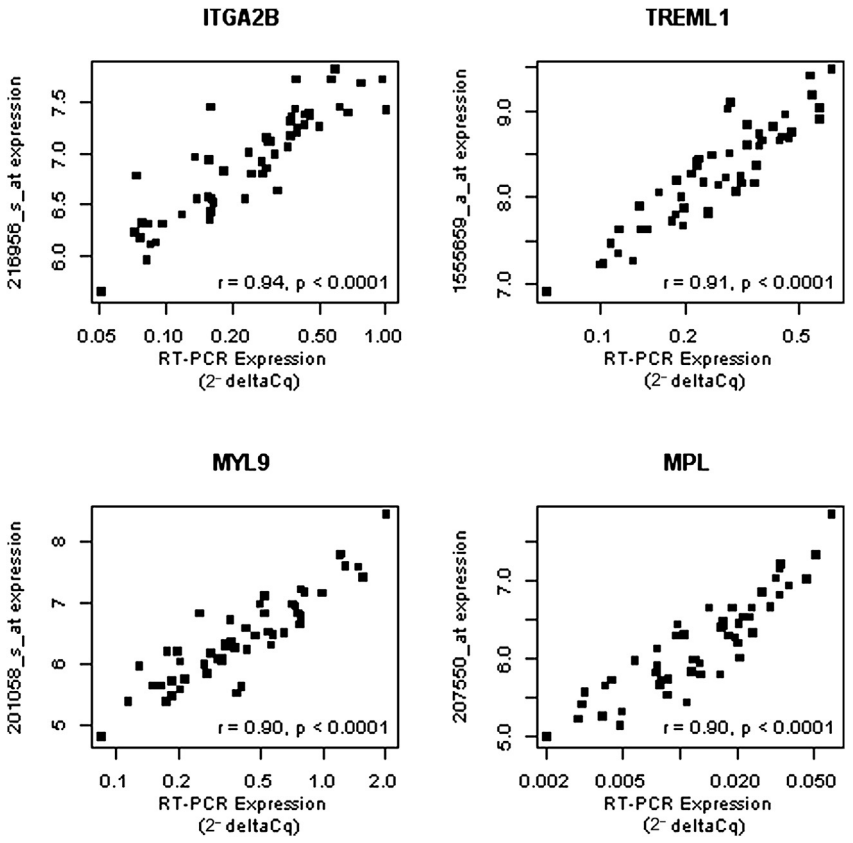

Figure 3 **RT-PCR–Based Assays Verify the Microarray-Based Gene Expression Values for ARS Genes**

Real-time polymerase chain reaction (RT-PCR) assays were designed to verify selected transcripts represented by the aspirin response signature (ARS) in the healthy volunteer validation cohort. The deltaCq for each assay was correlated with the robust multichip average–normalized probe set expression for the corresponding ARS gene using Pearson's correlation (see Online Table 2). For the 4 genes with the highest PCR versus microarray-based correlations (*ITGA2B*, *MYL9*, *TREML1*, and *MPL*), we plot the relative quantity ($2^{-\text{deltaCq}}$, x-axis, log-scale) versus the corresponding probe set expression (y-axis), correlation coefficient, and p value.

expressed as ΔCq (see the Online Appendix) and correlated with the corresponding microarray probe set or PFS using Pearson tests of correlation.

PLATELET PROTEOMIC DATASET ANALYSIS. See the Online Appendix for full details.

ANALYSES OF CATHGEN COHORTS. Logistic or Cox proportional hazards regression models were created in the case-control or observational cohort, respectively, to test for association between a factor and death or MI. Each model tested the factor alone as well as after controlling for baseline variables (Online Table 6) associated with the factor of interest. The assumption of proportional hazards for each Cox model was met. Odds ratios (ORs) (or hazard ratios), 95% confidence intervals (CIs), and p values are reported.

To assess the independent association between a factor and death or MI, logistic regression models were built on the combined CATHGEN cohorts by forcing Framingham risk factors (age, sex, smoking, diabetes, hypertension, and hyperlipidemia), African-American race, cohort, platelet count, and the presence of CAD (defined as a CAD index [15] >32 or a history of coronary artery bypass surgery, MI, or percutaneous coronary intervention) into the model and adding the factor score or individual probe set gene expression. To assess the incremental prognostic value of gene expression, we compared the performance of competing models (risk factors with or without factor or probe set expression), using the areas under the receiver-operating characteristic curve (16), the net reclassification index (using risk categories of <10%, 10% to 20%, and >20%) (17) or category-free net reclassification index (18), and the integrated discrimination improvement (17).

Results

Discovery and validation of a set of coexpressed genes in whole blood that correlate with platelet function on aspirin. In the discovery cohort (HV1), we identified 20 factors (numbered 1 to 20) (Online Table 1) representing sets of highly correlated, coexpressed genes. To test the hypothesis that 1 or more of these gene sets were associated with PFS on aspirin, we correlated each set with PFS in HV1

and identified "factor 14" (Fig. 1, Discovery Cohort) and "factor 3" ($r = 0.27$, $p = 0.05$). In the first validation cohort (HV2), we found a significant association between factor 14 and PFS, with the same strength and direction as observed in HV1 (Bonferroni-adjusted $p = 0.03$; Fig. 1, Validation Cohort #1), thus validating this association, but factor 3 was not associated with PFS in HV2. We further validated factor 14 with VerifyNow test results in the OPC cohort (Fig. 2). Thus, factor 14, which we named the "aspirin response signature" (ARS), was validated in 2 independent cohorts as a set of coexpressed genes associated with platelet function on aspirin.

To verify the microarray-based expression of the ARS transcripts, we selected 45 of the 60 genes (see Methods for selection criteria) for verification in whole-blood RNA from the HV2 cohort. Using real-time polymerase chain reaction (RT-PCR), 42 of 45 transcripts were significantly correlated with their microarray-based expression, with 16 of these 42 transcripts, including *ITGA2B*, *TREML1*, *MYL9*, and *MPL*, strongly ($r > 0.80$) correlating with microarray-based gene expression (Fig. 3, Online Table 2). For the majority of transcripts, there was concordance between both the RT-PCR and microarray correlations with PFS (Online Table 3, Online Fig. 1). Therefore, RT-PCR assays validate the microarray-based expression associations with PFS for most ARS transcripts.

ARS transcripts are primarily of platelet origin. We observed that the transcripts with the strongest correlations with PFS (Table 1) mapped to several well-known platelet transcripts: *ITGA2B*, *CLU*, *IGF2BP3*, *GP1BB*, and *SPARC*. On the basis of this observation, we hypothesized that transcripts represented by the ARS were of platelet origin. To test this hypothesis, we examined the overlap and enrichment of the 60 genes represented by the ARS with pre-defined gene sets specific to various peripheral blood cell types. Up to 24 of the 60 ARS genes significantly overlapped with platelet-specific or megakaryocyte-specific genes, whereas none overlapped with nonplatelet peripheral blood cell–type genes (Online Tables 4 and 5). Furthermore, in the CATHGEN cohorts, we found the strongest correlation between expression of the ARS and platelet count ($r = 0.41$, $p < 2 \times 10^{-16}$), with no strong positive correlations with any other peripheral blood cell type counts: white blood cells ($r = -0.01$, $p = 0.87$), lymphocytes ($r = -0.25$, $p = 1.2 \times 10^{-5}$), neutrophils ($r = 0.16$, $p = 0.01$), or monocytes ($r = 0.06$, $p = 0.27$).

To confirm the platelet origin of the ARS genes, we analyzed purified platelet lysates by label-free proteomics in the HV2 cohort. We identified 17 proteins from the ARS gene set in the proteomics dataset, of which 6 were associated with PFS, including *ITGA2B*, *ITGB3*, and *MYL9* (Table 2), all in the same direction as their corresponding transcripts. Therefore, from these data, we conclude that a large number of ARS transcripts originate in platelets and are thus reporting on a coexpressed pathway of platelet transcripts and proteins associated with platelet function on aspirin.

Because MPV is associated with platelet function (19) and the platelet origin of ARS transcripts, we assessed the extent to which the association between ARS and PFS was confounded by platelet volume or count. After controlling for MPV, the ARS remained significantly (adjusted regression coefficient for ARS = −0.5, standard error = 0.2, $p = 0.05$ for HV1; adjusted regression coefficient for ARS = −0.87, standard error = 0.4, $p = 0.03$ for HV2) associated with PFS. Furthermore, in HV1, in which platelet count and volume were both measured, the ARS remained significantly (-0.5 ± 0.2, $p = 0.04$) associated with PFS after their inclusion. Therefore, the association between ARS and platelet function is independent of other readily available platelet parameters, such as count and MPV.

Before the administration of aspirin, the ARS is not associated with platelet function. Because pre-aspirin platelet function is a strong predictor of post-aspirin platelet function (8), we tested the hypothesis that the aggregate expression of the ARS genes was correlated with native pre-aspirin PFS. In neither HV1 nor HV2 did we observe a correlation between the ARS and pre-aspirin PFS (Fig. 4). Despite the absence of a correlation with PFS before aspirin, the ARS genes were similarly coexpressed before and after aspirin exposure (Online Fig. 2). Therefore, although the ARS genes are highly correlated with one another before aspirin exposure, their aggregate expression does not appear to contribute to native pre-aspirin platelet function. Instead, the expression of the ARS genes specifically reflects platelet function on aspirin.

The ARS is an independent prognostic biomarker for cardiovascular events. Because of the association of the ARS with platelet function on aspirin and aspirin's role in preventing cardiovascular events, we tested the hypothesis that the ARS was associated with the risk for death or MI in 2 independent patient cohorts. In both the case-control and observational cohorts, the ARS was significantly associated with death or MI in univariate analyses (OR: 1.2; 95% CI: 1.04 to 1.4; $p = 0.04$; and hazard ratio: 1.4; 95% CI: 1.1 to 1.7; $p = 0.002$, respectively). The majority of the individual transcripts represented by the ARS were also associated with death or MI in both cohorts (Online Table 7).

To determine the extent to which the ARS or an individual probe set for *ITGA2B* was an independent prognostic biomarker for events, we combined the CATHGEN cohorts and found that the ARS (OR: 1.3; 95% CI: 1.1 to 1.5; $p = 0.001$) or the microarray-based expression of *ITGA2B* (probe set 206494_s_at; OR: 1.5; 95% CI: 1.2 to 1.8; $p = 0.0001$) were independently associated with death or MI after adjustment for Framingham risk factors (20), race, platelet count, and the presence of angiographic CAD.

To further assess the potential use of the ARS as a risk biomarker, we tested the hypothesis that the ARS or *ITGA2B* probe set expression would improve measures of discrimination. Compared with a model using clinical risk factors alone,

Table 1 Genes Represented by the ARS and Their Correlations With Platelet Function With Aspirin

Affymetrix Probe ID	Gene Symbol	Gene Description	Combined PFS Beta Coefficient*	Combined p Value
Factor 14	NA	NA	−0.76088	0.0017
Individual factor 14 transcripts				
208782_at	*FSTL1*	Follistatin-like 1	−1.6579	0.0003
201059_at	*CTTN*	Cortactin	−1.2817	0.0015
201906_s_at	*CTDSPL*	CTD (RNA polymerase II, polypeptide A) small phosphatase–like	−1.3795	0.0025
1555659_a_at	*TREML1*	Triggering receptor expressed on myeloid cells–like 1	−1.0767	0.0034
212667_at	*SPARC*	Secreted protein, acidic, cysteine-rich (osteonectin)	−1.214	0.0048
216956_s_at	*ITGA2B*	Integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	−1.0689	0.0048
230942_at	*CMTM5*	CKLF-like MARVEL transmembrane domain containing 5	−1.1641	0.0061
57588_at	*SLC24A3*	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	−1.3053	0.0063
207550_at	*MPL*	Myeloproliferative leukemia virus oncogene	−0.931	0.0066
219090_at	*SLC24A3*	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	−1.1123	0.0080
208791_at	*CLU*	Clusterin	−0.9584	0.0085
206494_s_at	*ITGA2B*	Integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	−0.7279	0.0087
227189_at	*CPNE5*	Copine V	−1.2062	0.0088
220496_at	*CLEC1B*	C-type lectin domain family 1, member B	−1.2077	0.0090
206493_at	*ITGA2B*	Integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	−0.8966	0.0094
206049_at	*SELP*	Selectin P (granule membrane protein 140kDa, antigen CD62)	−1.1642	0.0104
203819_s_at	*IGF2BP3*	Insulin-like growth factor 2 mRNA binding protein 3	−1.2947	0.0123
225354_s_at	*SH3BGRL2*	SH3 domain binding glutamic acid-rich protein–like 2	−1.0895	0.0146
207808_s_at	*PROS1*	Protein S (alpha)	−1.1049	0.0174
207206_s_at	*ALOX12*	Arachidonate 12-lipoxygenase	−0.8756	0.0207
212813_at	*JAM3*	Junctional adhesion molecule 3	−1.0454	0.0215
1560262_at	*LRRC32*	Leucine-rich repeat-containing 32	−0.9376	0.0226
204628_s_at	*ITGB3*	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	−0.968	0.0242
214146_s_at	*PPBP*	Proplatelet basic protein (chemokine [C-X-C motif] ligand 7)	−0.713	0.0243
211026_s_at	*MGLL*	Monoglyceride lipase	−1.0027	0.0249
208792_s_at	*CLU*	Clusterin	−0.8122	0.0266
201108_s_at	*THBS1*	Thrombospondin 1	−0.9169	0.0276
201058_s_at	*MYL9*	Myosin, light chain 9, regulatory	−0.5909	0.0287
206390_x_at	*PF4*	Platelet factor 4 (chemokine [C-X-Cmotif] ligand 4)	−0.9017	0.0296
206655_s_at	*GP1BB*	Glycoprotein Ib (platelet), beta polypeptide	−0.8934	0.0317
209651_at	*TGFB1I1*	Transforming growth factor beta 1–induced transcript 1	−0.7618	0.0326
207414_s_at	*PCSK6*	Proprotein convertase subtilisin/kexin type 6	−0.8566	0.0351
200665_s_at	*SPARC*	Secreted protein, acidic, cysteine-rich (osteonectin)	−0.8261	0.0410
212077_at	*CALD1*	Caldesmon 1	−0.5688	0.0505
203817_at	*GUCY1B3*	Guanylate cyclase 1, soluble, beta 3	−0.8511	0.0546
227088_at	*PDE5A*	Phosphodiesterase 5A, cGMP-specific	−0.918	0.0571
226152_at	*TTC7B*	Tetratricopeptide repeat domain 7B	−0.7986	0.0594
206167_s_at	*ARHGAP6*	Rho GTPase-activating protein 6	−0.8437	0.0677
37966_at	*PARVB*	Parvin, beta	−0.7708	0.0717
208601_s_at	*TUBB1*	Tubulin, beta 1	−0.5959	0.0736
204115_at	*GNG11*	Guanine nucleotide–binding protein (G protein), gamma 11	−0.5622	0.1229

Continued on the next page

Table 1 Continued

Affymetrix Probe ID	Gene Symbol	Gene Description	Combined PFS Beta Coefficient*	Combined p Value
241133_at	*PRSS1*	Protease, serine, 1 (trypsin 1)	−0.4814	0.1243
203680_at	*PRKAR2B*	Protein kinase, cAMP dependent, regulatory, type II, beta	−0.5049	0.1365
205442_at	*MFAP3L*	Microfibrillar-associated protein 3–like	−0.4724	0.1385
212151_at	*PBX1*	Pre-B-cell leukemia transcription factor 1	−0.6059	0.1729
212573_at	*ENDOD1*	Endonuclease domain–containing 1	−0.7276	0.1735
230690_at	*TUBB1*	Tubulin, beta 1	−0.578	0.1864
230645_at	*FRMD3*	FERM domain–containing 3	−0.6391	0.2102
225974_at	*TMEM64*	Transmembrane protein 64	0.38321	0.2227
1553842_at	*BEND2*	Chromosome X open reading frame 20	−0.5657	0.2258
228708_at	*RAB27B*	RAB27B, member Ras oncogene family	−0.4836	0.2512
227180_at	*ELOVL7*	ELOVL family member 7, elongation of long-chain fatty acids (yeast)	−0.3943	0.2823
212148_at	*PBX1*	Pre-B-cell leukemia transcription factor 1	−0.3139	0.2970
203414_at	*MMD*	Monocyte to macrophage differentiation–associated	−0.4287	0.3236
1552773_at	*CLEC4D*	C-type lectin domain family 4, member D	0.37545	0.3543
222717_at	*SDPR*	Serum deprivation response (phosphatidylserine binding protein)	−0.3009	0.3830
224823_at	*MYLK*	Myosin, light chain kinase	−0.2911	0.4644
214974_x_at	*CXCL5*	Chemokine (C-X-C motif) ligand 5	−0.1621	0.5011
229778_at	*C12ORF39*	Chromosome 12 open reading frame 39	−0.2032	0.5020
235331_x_at	*PCGF5*	Polycomb group ring finger 5	0.22781	0.5470
212651_at	*RHOBTB1*	Rho-related BTB domain–containing 1	−0.2395	0.5755
206110_at	*HIST1H3H*	Histone cluster 1, H3h	−0.2021	0.5827
215779_s_at	*HIST1H2BG*	Histone cluster 1, H2bg	−0.2623	0.5896
207815_at	*PF4V1*	Platelet factor 4 variant 1	−0.0927	0.6139
226188_at	*LGALSL*	Lectin, galactoside-binding–like	0.23728	0.6142
221556_at	*CDC14B*	CDC14 cell division cycle 14 homolog B (*S. cerevisiae*)	−0.2127	0.6530
207156_at	*HIST1H2AG*	Histone cluster 1, H2ag	−0.1534	0.6882
210387_at	*HIST1H2BG*	Histone cluster 1, H2bg	−0.1494	0.6906
225166_at	*ARHGAP18*	Rho GTPase–activating protein 18	0.15541	0.7353
206272_at	*RAB4A*	RAB4A, member Ras oncogene family	0.09962	0.7967
210986_s_at	*TPM1*	Tropomyosin 1 (alpha)	0.08749	0.8404
227451_s_at	*C6ORF79*	Chromosome 6 open reading frame 79	−0.0134	0.9791

*The beta coefficient for the expression of either the aggregate expression of the ARS or each probe set represented by the ARS using the combined HV1 and HV2 datasets from a regression model containing gene expression and cohort (HV1 vs. HV2) with corresponding p values.

ARS = aspirin response signature; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; CTD = carboxyterminal domain; GTPase = guanosine triphosphatase; HV1 = healthy volunteer discovery cohort; HV2 = healthy volunteer validation cohort; ID = identifier; mRNA = messenger ribonucleic acid; NA = not applicable; PFS = platelet function score; RNA = ribonucleic acid.

the inclusion of the ARS improved most measures of risk discrimination (Table 3, Fig. 5A, Online Table 8). Inclusion of *ITGA2B* probe set expression significantly improved all measures of discrimination (Table 3, Fig. 5B, Online Table 8). Thus, the ARS or the expression of an individual ARS transcript such as *ITGA2B* was an independent prognostic biomarker for risk for death or MI.

Discussion

We used aspirin as a probe to identify novel genes and biomarkers associated with platelet function and cardiovascular events. We hypothesized that administering aspirin while assaying the blood transcriptome might identify sets of genes that are related to aspirin's cardioprotective effect. We identified a set of platelet-enriched, coexpressed genes and proteins, the ARS, that was reproducibly associated with platelet function in response to aspirin. When tested as a prognostic biomarker, the ARS or an individual ARS transcript (e.g., *ITGA2B*) independently and incrementally predicted the risk for death or MI compared with traditional risk factors. Our data show that: 1) the genomic response to a pharmacological "challenge" with aspirin can reveal genes that underlie platelet function on aspirin and mechanisms responsible for death or MI; and 2) whole-blood RNA profiling may identify novel biomarkers that discriminate individuals at heightened risk for death or MI.

Transcripts associated with platelet function on aspirin are associated with cardiovascular events. We found neither an association between the ARS and the presence of

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Table 2 Aspirin Response Signature Proteins Identified in Platelet Protein and Their Correlations With PFS on Aspirin

Protein Name	Correlation With PFS	p Value
TBB1	−0.32	0.02
GP1BB	−0.29	0.03
ITA2B	−0.29	0.03
ITB3	−0.28	0.04
MYL9	−0.27	0.05
RB27B	−0.26	0.06
LEGL	−0.24	0.08
TSP1	−0.24	0.08
CALD1	−0.22	0.11
SRC8	−0.21	0.12
SH3L2	−0.20	0.16
CXCL7	−0.20	0.15
SDPR	−0.18	0.19
PLF4	−0.18	0.20
SPRC	−0.14	0.31
PDE5A	−0.09	0.49
CLUS	0.06	0.67

PFS = platelet function score.

CAD nor overlap between ARS genes previously associated with CAD (1,6). Instead, we found that the ARS was associated with death or MI after controlling for CAD and CAD risk markers. These findings highlight a unique and novel role that the biological pathway represented by ARS genes has in the development of cardiovascular events, independent of CAD. We conclude that the biology of aspirin is complex and involves additional mechanisms beyond inhibiting platelet cyclo-oxgenase-1, and some of these mechanisms underlie risk for cardiovascular events.

A novel and translatable biomarker of platelet function in response to aspirin and the risk for cardiovascular events. Clinicians currently need a readily available biomarker for the response to aspirin. Despite the availability of platelet function assays, their widespread use is severely constrained by the need for specialized equipment and trained personnel. Point-of-care tests are available but require testing to be completed within hours of phlebotomy; thus, they are out of reach for the vast majority of outpatients on aspirin. Furthermore, most patients taking aspirin for chronic prevention are outpatients in whom results at the point of care are not required. Instead, testing in central laboratories, as is common for low-density lipoprotein cholesterol for statins, would be sufficient for determining aspirin response in the outpatient setting. Because of the coexpressed nature of the ARS genes, several individual transcripts (Table 1) correlated best with platelet function. We demonstrated that PCR for individual transcripts could be used in lieu of microarrays (Fig. 3, Online Table 2) for many ARS genes, thus demonstrating the feasibility of a blood-based diagnostic test.

Whole-blood RNA testing is a well-established diagnostic testing platform. For cardiac allograft rejection and CAD diagnosis, whole-blood microarray analyses were both transitioned to a PCR-based platform (6,7): AlloMap (XDx, Inc., Brisbane, California) and Corus CAD (CardioDx, Inc., Palo Alto, California), respectively. AlloMap has been approved by the U.S. Food and Drug Administration, and both are covered by major insurers. Therefore, there is a feasible path for blood-based RNA biomarkers to clinical adoption, Food and Drug Administration approval, and insurance coverage.

Peripheral blood gene expression profiling reveals coexpressed transcripts of platelet origin associated with platelet function in response to aspirin. The genes underlying variable platelet function on aspirin have been difficult to identify (21) or explain a small portion of the observed variability (22). We hypothesized that whole-blood RNA profiling, which de facto contains platelet transcripts, would yield biological pathways important for the response to aspirin. We demonstrated that the transcripts represented by the ARS are likely of platelet origin (Online Tables 4 and 5). When we analyzed platelet-enriched protein, we not only

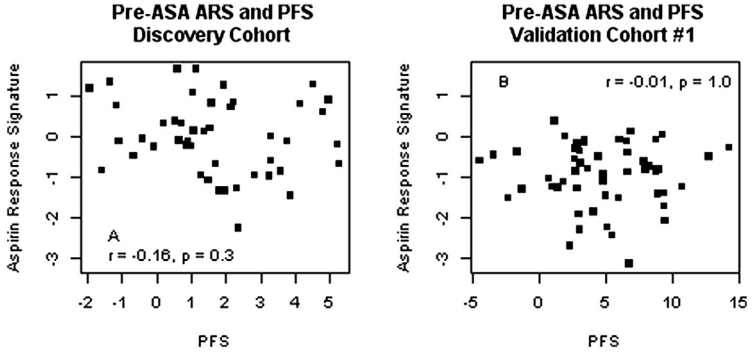

Figure 4 A Set of Coexpressed Peripheral Blood Genes Does Not Correlate With Native Pre-Aspirin Platelet Function

The aggregate expression of coexpressed genes is plotted against the platelet function before the administration of aspirin (ASA) in the healthy volunteer discovery cohort **(A)** (n = 45) and the healthy volunteer validation cohort **(B)** (n = 50). Pearson's correlation coefficients and p values are reported. ARS = aspirin response signature; PFS = platelet function score.

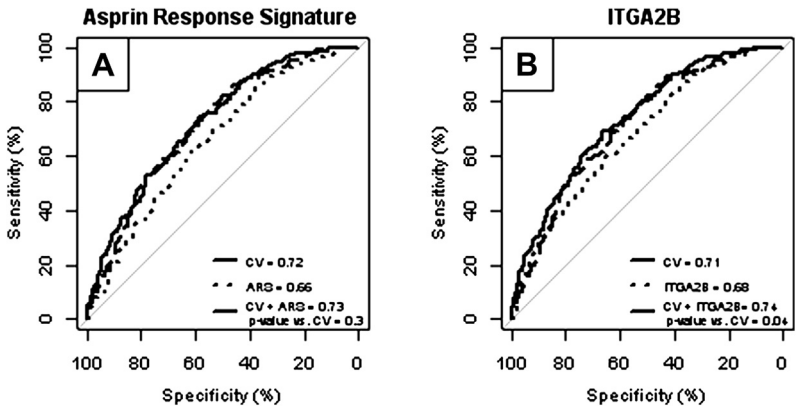

Figure 5 **Peripheral Blood Gene Expression Adds Additional Prognostic Information for Death or MI**

Patients in the case-control and observational cohorts were combined and analyzed with respect to death or myocardial infarction (MI) outcomes. The receiver-operating characteristic curves were plotted for predictive models containing cardiovascular risk factors, platelet count, the presence of coronary artery disease, cohort (collectively, CV) and gene expression for the aspirin response signature **(A)** or one of its genes ITGA2B **(B)**, or both were compared. The probe set 216956_s_at represents *ITGA2B* gene expression. ARS = aspirin response signature.

confirmed the well-known roles of *ITGA2B* and *ITGB3*, but also ascribe new roles to many other platelet genes, including *MYL9*, *CLU*, *PPKAR2B*, *TREML1*, and *CTTN*, with respect to platelet function on aspirin and cardiovascular events. Additionally, recent genomewide association studies identified a *PEAR1* polymorphism associated with platelet *PEAR1* levels and platelet function on aspirin (22). We excluded the probe set (228618_at) mapping to *PEAR1* because its variance (0.21) was below our variance criterion (0.25; see the Methods section). However, in a post-hoc analysis, *PEAR1* expression was strongly correlated (r = 0.9) with ARS levels. Therefore, our approach identified previously known and novel platelet genes associated with platelet function in response to aspirin.

We observed an association between ARS and platelet function only after the administration of aspirin, suggesting that the latent effect of ARS genes on platelet function is unmasked in response to aspirin. Consistent with these findings, when we stratified the CATHGEN cohort by aspirin use, we observed that the association between the ARS and death or MI was higher in those using aspirin at the time of catheterization (OR: 1.4 vs. 1.1 in aspirin users vs. nonusers). We hypothesize that the molecular mechanisms represented by the ARS contributes minimally to native platelet function in the absence of aspirin. By contrast, when platelet cyclooxygenase-1, a protein not represented by the ARS, is suppressed by 325 mg/day aspirin dosing (23), the effects of these platelet-enriched genes is revealed such that the resulting

Table 3 **Measures of Discrimination With and Without Inclusion of Gene Expression Profiles**

Measure	Traditional Risk Factors	Traditional Risk Factors + ARS	Traditional Risk Factors + 216956_s_at† (*ITGA2B*)
Area under ROC curve	0.72	0.73	0.74
95% CI	0.68–0.76	0.69–0.77	0.70–0.78
p Value*	NA	0.3	0.04
Net reclassification index (<10%, 10%–20%, >20%)	—	0.06	0.12
95% CI		0.02–0.10	0.07–0.17
p Value		0.005	$<1 \times 10^{-5}$
Net reclassification index (category free)	—	0.31	0.37
95% CI		0.15–0.47	0.21–0.54
p Value		2×10^{-4}	8.7×10^{-6}
Integrated discrimination improvement	—	0.01	0.03
95% CI		0.002–0.02	0.02–0.05
p Value		0.006	2×10^{-5}

*All p values are for comparisons with "traditional risk factors" model, which includes age, sex, African-American race, smoking, diabetes, hypertension, hyperlipidemia, cohort, and the presence of coronary artery disease. †The 216956_s_at probe set represents *ITGA2B* gene expression on the Affymetrix microarray.

CI = confidence interval; ROC = receiver-operating characteristic; other abbreviations as in Table 1.

level of platelet function is then determined by the ARS. Alternatively, aspirin exposure may alter the genomic and protein content of circulating platelets. The precise mechanism by which platelet function on aspirin is related to the expression of the ARS genes and proteins on aspirin is the subject of ongoing work.

Study limitations. Several limitations deserve consideration. Neither platelet function nor MPV was measured in CATHGEN. Therefore, we cannot know whether heightened ARS levels altered platelet function or volumes in addition to an increased risk for death or MI. To our knowledge, large cohorts with platelet function, banked RNA, longitudinal follow-up, and a sufficient number of events are not available. Furthermore, in our discovery and validation cohorts, the association of the ARS with PFS was independent of platelet count and MPV, suggesting that the ARS provides an independent parameter of platelet function that underlies cardiovascular events. Second, although there was no association between the ARS and modifiable risk factors (e.g., diabetes, hyperlipidemia, or hypertension), because we did not assess the degree to which these risk factors were controlled, we do not know whether addressing these risk factors could modulate ARS levels. Finally, the comparison of the ARS gene set with that of platelets, megakaryocytes, and platelet proteomics analyses demonstrate that the top ARS genes correlative of platelet function on aspirin were of platelet origin. However, some ARS genes (e.g., *TTC7B*, *FSTL1*) are also expressed in nonplatelet cell types, suggesting that mechanism(s) represented by ARS genes may involve more than just platelets.

Conclusions

We used aspirin as a probe in conjunction with RNA profiling and identified novel biomarkers that identify patients at highest risk for death or MI independent of clinical risk factors.

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Reprint requests and correspondence: Drs. Deepak Voora or Geoffrey Ginsburg, Institute for Genome Sciences & Policy, Duke University, 101 Science Drive, Durham, North Carolina 27710. E-mail: deepak.voora@duke.edu OR geoffrey.ginsburg@duke.edu.

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Key Words: aspirin ■ biomarkers ■ genes ■ myocardial infarction ■ platelets.

APPENDIX

For an expanded Methods section, and supplementary figures and tables and their legends, please see the online version of this article.